

where Y is the tyrosine at amino acid number 639 in the T7 RNA polymerase protein. The same consensus sequence is observed in the SP6 RNA polymerase and T3 RNA polymerase proteins where a K (K=lysine) is succeeded by 7 amino acids and a Y G (G=glycine). In SP6 RNA polymerase the Y is at amino acid number 631 in the polypeptide chain, and in T3 RNA polymerase it is at amino acid number 573. By mutating the codon for Y631 in SP6 RNA polymerase such that a phenylalanine is at this position, the expected phenotypic change was realized.

In summary, one may locate the corresponding mutation site in other RNAPs by aligning the amino acid sequence of a T7-like RNAP, chosen from among T7, T3,  $\phi$ I,  $\phi$ IIH, W31, ghl, Y, A1122, SP6 and mitochondrial RNAPs, against the conserved motif given above and identifying which position corresponds to the Y639 position in T7 RNAP.

As stated above, the conserved motif is also present in class I DNAPs. While a structure of T7 RNAP complexed with NTP is not available, the structure of the homologous Klenow fragment of DNAP I with dNTP has been obtained (Beese, et al., 1993). This structure demonstrated that the amino acids encompassed within the above-mentioned conserved motif (i.e., amino acid residues 758 to 767 of *E. coli* DNAP I) are in proximity to the deoxyribose sugar of the dNTP, so that it is reasonable that mutations within this motif might affect the ability of a DNAP to discriminate between dNTPs and rNTPs. However, one of the present inventors found that when a mutation was made which changed the amino acid at the position in a class I DNAP corresponding to the Y639 mutation in T7 RNAP, the mutant DNAP retained enzymatic activity, but did not have a reduced discrimination for rNTPs compared to dNTPs. Thus, it is not obvious what

mutations, if any, would result in a class I DNAP having reduced discrimination for rNTPs vs. dNTPs, even if it is reasonable to assume that such a mutation would occur within the above-mentioned conserved motif (residues 758 to 767 of *E. coli* DNAP I) which the structure shows to be in proximity to the dNTP.

Because the structure of the Klenow fragment of DNAP I complexed with dNTP was determined (Beese, et al., 1993), researchers have believed that the homologous conserved motif of T7 RNAP (i.e., amino acid residues 631-640) is likely to be in proximity to the ribose moiety of an NTP, as the case for the DNAP I. Nevertheless, prior to the work of the present invention, it was not possible to know which mutation, if any, might result in a reduced discrimination for dNTPs vs. rNTPs. Since the Y639 mutation of T7 RNAP was identified, as presented herein, one of the present inventors has modeled NTP in T7 RNAP (Huang, et al., submitted for publication) based on the structures of the homologous Klenow fragment of DNAP I complexed with dNTP (Beese, et al., 1993) and of RT complexed with primer-template (Jacabo-Molina, et al., 1993). Models based on either structure agree in placing the ribose close to Y639, and in revealing no other side chain capable of discriminating the hydrogen bonding character of the 2'-substituent within 5 angstroms of the 2'-group of the NTP. Thus, the model is consistent with our results related to a reduced discrimination of Y639 RNAP mutants for dNTPs vs. rNTPs, even though additional studies (Huang, et al., submitted for publication) have determined that the hydrogen bonding character is not the only factor involved in dNTP/rNTP discrimination.

Less is known about non-T7-type RNAPs. For many, the amino acid sequences are not known. Non-phage-encoded host bacterial RNA polymerases are complex multi-subunit proteins. A nucleotide polymerization site has been localized in the  $\beta$  subunit of *E. coli* RNA polymerase although participation of other subunits is not ruled out. In order to determine the site in a non-T7-like RNAP which would result in a reduced dNTP/rNTP discrimination, one would first use the above-described procedure of alignment

to determine if the

... K — — — — — Y G ... motif was present. If so, it may be possible to obtain the desired mutation in the same manner as for T7-like RNAPs. However, if the conserved motif is not present, one may obtain the desired mutation with greater difficulty by random mutagenesis and enzyme assay screening in order to find a change or changes that result in reduced dNTP/rNTP discrimination.

Once one has determined where the corresponding Y639 site is in the polymerase one wishes to mutate, one would use standard methods in the art of molecular biology to create an amino acid substitution. As disclosed above, a conservative substitution is preferable. For example, a substitution of a phenylalanine for a tyrosine is most preferable. The Examples below disclose a method for creating a mutant polymerase, but one of skill in the art will realize that there are many substitute methods of equal effectiveness.

#### Methods of the Present Invention

In one embodiment, the present invention is a method for using a mutant polymerase for synthesizing *in vitro* a nucleic acid molecule which comprises at least one non-